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Atty. Dkt. No. 041673-2053

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Tuszynski, Mark H.

Title: METHODS FOR THERAPEUTIC USE OF BRAIN DERIVED NEUROTROPHIC FACTOR IN THE ENTORHINAL CORTEX

Appl. No.: 10/039,078

Filing Date: 12/31/2001

Examiner: Anne Marie Falk

Art Unit: 1632

CERTIFICATE OF FACSIMILE TRANSMISSION
I hereby certify that this paper is being facsimile transmitted to the United States Patent and Trademark Office, Alexandria, Virginia on the date below.

Rachel Caputo
(Printed Name)

(Signature)

November 29, 2005
(Date of Deposit)

DECLARATION OF MARK H. TUSZYNSKI UNDER 37 CFR 1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Mark H. Tuszynski, declare as follows:

1. I am an inventor of the invention claimed in U.S. Patent No. 6,451,306, together with my co-inventor, Fred Gage.

2. While Dr. Gage made a significant contribution to the invention claimed in the '306 Patent (methods for *ex vivo* gene therapy by grafting neurotrophin-encoding donor cells into the cholinergic forebrain), he did not make an inventive contribution to the presently claimed invention (methods for gene therapy of cortical tissues, including by *in vivo* delivery of a neurotrophin-encoding expression vector to the cortices). The latter invention was conceived by me and developed without participation by, or input from, Dr. Gage. Therefore, I am the sole inventor of the presently claimed invention.

Atty. Dkt. No. 041673-2053

I hereby declare that all statements made herein of my known knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements are so made punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: Nov 22, 2005

Mark H. Tuszynski

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November 29, 2005
(Date of Deposit)

DECLARATION OF MARK H. TUSZYNSKI UNDER 37 CFR 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. I, Dr. Mark H. Tuszynski, am the inventor of the invention that is claimed in this application. I am employed as a Professor of Neurosciences and the Director of the Neural Repair Center at the University of California, San Diego. This declaration concerns experiments conducted under my direction with respect to the use of brain derived neurotrophic factor (BDNF), delivered in a recombinant expression vector to brain tissue, as an agent to protect neurons from cell death and degeneration.

2. To determine whether BDNF can prevent the death of entorhinal cortical neurons, we transected the perforant path in adult Fischer 344 rats, thereby interrupting projections from entorhinal cortex to the hippocampus. This lesion typically induces death in 20-25% of layer II entorhinal cortical neurons. Eight rats each received three injections into the right entorhinal cortex of lentiviral vectors constitutively expressing human BDNF under control of the CMV/ß-actin hybrid promoter. Six control rats received injections of lentiviral vectors expressing the reporter gene green fluorescent protein (GFP).

3. Four days after treatment, right-sided perforant path lesions were made in the treated rats. Two weeks after placement of perforant path lesions, the number of surviving neurons in the entorhinal cortex was quantified using unbiased stereological methods. Animals that underwent lesions and injections of control GFP-expressing vector exhibited a significant 20% loss of neurons in layer II entorhinal cortex compared to the contralateral, intact side of the brain (ANOVA $p<0.0001$). In contrast, BDNF-treated rats demonstrated essentially complete protection from retrograde cell death ($p<0.001$ compared to GFP-injected controls). Cell shrinkage (atrophy) was also significantly ameliorated by BDNF-treatment: mean cell size was reduced to 85% of normal values in GFP-injected lesioned controls (ANOVA $p<0.0001$), but retained 94% of intact values after lentiviral BDNF treatment ($p<0.001$ compared to GFP-injected controls). These data show that BDNF prevents lesion-induced death and ameliorates atrophy of entorhinal cortical neurons.

3. To determine whether BDNF neuroprotection extends to a direct Alzheimer's disease-related mechanism of degeneration, we first examined BDNF effects *in vitro* on ß-amyloid-induced cell toxicity in primary entorhinal cortical neuronal cultures. Entorhinal cortical neurons were dissected from postnatal-day-3 rats and established as primary dissociated cell cultures. Addition of ß-amyloid 1-40 or 1-42 peptide induces the death of neurons in cell culture within 24 hours, a finding replicated in this experiment. Addition of recombinant BDNF in the culture medium concurrent with ß-amyloid (Aß) peptide exposure conferred complete protection from Aß toxicity in a dose-dependent manner. BDNF specifically prevented Aß-induced cell death rather than non-specifically increasing the total number of neurons via general trophic actions, since addition of BDNF to entorhinal cultures in the absence of Aß did not increase total neuronal number. These data show that BDNF protects entorhinal neurons from Aß toxicity.

4. To directly test the effects of BDNF in an *in vivo* model of neuronal degeneration resulting from Alzheimer's disease-related pathology, we examined transgenic mice bearing the double Swedish (671_{KM_NL}) and Indiana (717_{V_F}) amyloid mutations. These mice develop diffuse cortical plaques and cognitive decline by age 4 months, and progressive cell loss in the entorhinal cortex beginning at age 2-3 months. Lentivirus expressing BDNF-GFP (n=8) or GFP alone (n=8) was injected into the entorhinal cortices of the mutant Aß transgenic mice bilaterally at age 6 months. A parallel group of age-matched wild-type littermates underwent sham surgery (n=4) or injection of lentivirus expressing GFP into the entorhinal cortices bilaterally (n=4). One month later, spatial memory was tested in the Morris water maze. The performance of sham-operated

and GFP-injected wild-type subjects did not differ ($p=0.9$), and they were combined for subsequent statistical analysis.

5. Over 5 successive daily blocks of trials, significant amelioration of spatial memory deficits occurred in BDNF-injected animals compared to GFP-injected transgenics ($p<0.05$); the performance of the BDNF-treated-transgenics did not differ from the wild-type mice. These data confirm that, as described with respect to treated, aged (but otherwise healthy) animals in the present patent application at paragraphs 0044-0051, BDNF ameliorates spatial memory loss, here in transgenic mice overexpressing amyloid mutations related to Alzheimer's disease.

6. Transgenic mice were also tested on a fear-conditioning paradigm that measured both hippocampal-dependent (context) and hippocampal-independent (cue) learning. On Day 1, three pairs of a tone-shock were performed in an operant chamber, and on Day 2 fear conditioning was measured as a function of freezing in the same context or with the same cue. In the hippocampal-dependent (contextual) aspect of the task, APP mutant mice were impaired relative to wild-type littermates, and BDNF treatment ameliorated the deficit ($p<0.05$; Fig 4c). Hippocampal-independent (cue) fear conditioning was not impaired in either transgenic mouse group.

7. Thus, BDNF generated recovery of memory and preservation of skills dependent on integrity of the entorhinal/ hippocampal complex in APP transgenic mice. Further, these results are consistent with the observation made in the present patent application that the disclosed

treatment can produce beneficial responses not only in the entorhinal cortex, but also in the hippocampus (see, e.g., Specification at paragraph 0007). To my knowledge, responses to BDNF in these cortical tissues have never before been reported.

I hereby declare that all statements made herein of my known knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements are so made punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: Nov 22, 2005

Mark H. Tuszynski

Respectfully submitted,

Date 11-29-2005

By

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